INVESTIGATION OF ETHANOL, ACETONE AND AQUEOUS *Azadirachta indica* A. Juss (NEEM) EXTRACTS AGAINST *Plasmodium falciparum*

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by

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LIST OF EQUATIONS

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LIST OF SYMBOLS

~	approximately
%	per cent
°C	degree Celsius
=	equal
±	plus, minus
<	less than
≤	less than or equal to
>	more than
≥	more than or equal to
× g	gravitation force
µm	micromolar
µg/mL	microgram per milliliter
µL	microliter
Cm	centimeter
dH_20	distilled water
e.g.	for example,
G	gram
i.e.	that is
mg/kg	milligram per kilogram
mg/L	milligram per liter
mg/mL	milligram per milliliter
mL	milliliter
mM	millimolar
Nm	nanometer
nM	nanomolar

ppm	part per million
pH	potential of hydrogen
v/v	Volume per volume

LIST OF ABBREVIATIONS

ACT	Artemisinin-based Combination Therapy
Ag	silver
$AgNO_3$	silver nitrate
AgNPs	silver nanoparticles
Au	gold
BCG	bacillus calmette–guérin
BLASTn	Basic Local Allignment Search Tool nucleotide
bp	base pair
BSC	Biosafety Cabinets
BSLT	brine shrimp lethality test
CC_{50}	cytotoxicity concentrations
CE-MS	Capillary electrophoresis-Mass Spectrometry
CQ	chloroquine
CTAB	Cetyl Trimethylammonium Bromide
DDT	dichlorodiphenyltrichloroethane
dNTP	deoxynucleoside triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European medicines agency
ERAR	emergency response to artemisinin resistance
FCR	Folin- Ciocalteu Reagent
FRIM	Forest Research Institute Malaysia
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
FTIR	Fourier-transform infrared spectroscopy
GAE	Gallic acid equivalent
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GTS	Global Technical Strategy

HCQ	hydroxychloroquine
HeLa	Cervical cancer cell line
HUVEC	Human umbilical vein endothelial cells
HRP2	histidine-rich protein 2
IC_{50}	inhibitory concentrations
IRAB	fractionated Neem leaves extract
iRBC	infected red blood cells
IRS	indoor residual spraying
ITS	Internal
LC_{50}	lethal concentrations
LC-MS	Liquid chromatography-Mass Spectrometry
MCF 7	Breast cancer cell line
MFQ	mefloquine
MRSA	methicillin-resistant staphylococcus aureus
MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NIH_3T	Fibroblast cell line
NIST	National Institute Standard and Technology
NPs	nanoparticles
NRAs	national regulatory authorities
OSCC	oral squamous cell carcinoma
PBMCs	Peripheral blood mononuclear cells
PBO	piperonyl butoxide
PCR	Polymerase Chain Reaction
PdI	polydispersity index
PES	Polyethersulfone
PfDHFR-TS	*Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase
pfHRP2	*Plasmodium falciparum* histidine-rich protein 2
pLDH	Plasmodium lactate dehydrogenase
Pyr	pyrimethamine
QE	Quercetin equivalent
QN	quinine

QNX	quinacrine
RDTs	rapid diagnostic test
RNAs	Ribonucleic acids
ROS	reactive oxygen species
SM	severe malaria
SP	sulphadoxine-pyrimethamine
TE	Tris-EDTA
TBE	Tris/Borate/EDTA
TFC	total flavonoid content
TPC	total phenolic content
TPC	Total phenolic content
TNS	Trypsin neutralization solution
T-25	Culture flask 25 cm
WHO	World Health Organization

LIST OF APPENDICES

KAJIAN EKSTRAK ETANOL, ASETON DAN AKUES *Azadirachta indica* A. Juss (SEMAMBU) TERHADAP *Plasmodium falciparum*

ABSTRAK

Malaria merupakan masalah kesihatan awam yang telah menyebabkan morbiditi dan mortaliti tahunan yang ketara disebabkan oleh kemunculan parasit tahan ubat. Oleh itu, keperluan terhadap agen antimalaria baharu dengan mekanisma tindakan baharu dan potensi terapeutik yang luas adalah sangat penting. Dalam kajian ini, *Azadirachta indica* A. Juss (Semambu) yang merupakan remedi herba tradisional telah dipilih disebabkan oleh kekurangan data tentang sifat antimalaria Semambu yang terdapat di Malaysia. Oleh itu, kajian ini adalah bertujuan untuk menentukan secara *in vitro*, aktiviti antimalaria menggunakan ekstrak kasar daun pokok Semambu di Malaysia terhadap strain *Plasmodium falciparum* adaptasi makmal yang sensitif (3D7) dan tahan klorokuin (W2). Pertama, daun yang telah dikumpul ditaksonomikan oleh Institut Penyelidikan Perhutanan Malaysia (FRIM) dan kemudian disahtulenkan secara molekul melalui pencapjarian sebelum diekstrak dengan menggunakan larutan etanol, aseton dan akueus diikuti seterusnya oleh penyaringan fitokimia. Asai penyingkiran radikal DPPH digunakan untuk menentukan aktiviti oksida sebelum diteruskan dengan pemprofilan GC-MS. Ujian kemautan udang brin (BSLT) and asai 3-(4, 5-dimetiltiazol-2-il)-2, 5-difeniltetrazolium bromida (MTT) melawan sel primer endotelial vena umbilikal manusia (HUVEC) digunakan sebagai ujian ketoksikan. Kemudian, aktiviti antimalaria ditentukan menggunakan asai malaria berasaskan kependarfluoran hijau SYBR I terhadap kedua-dua strain. Kajian ini diteruskan dengan mensintesis secara mesra alam, zarah nanoperak (AgNPs) bagi Semambu dan sifat antimalarianya turut dikaji. Data kami menunjukan bahawa ekstrak kasar yang terhasil

oleh aseton (15.55 ± 0.04%) and etanol (9.20 ± 0.05%) menggunakan kaedah pengekstrakan Soxhlet menghasilkan ekstrak lebih banyak berbanding larutan akueus (5.86 ± 0.06%). Walaubagaimanapun, ekstrak kasar larutan akueus didapati mengandungi jumlah sebation fenolik dan flavonoid yang lebih tinggi berbanding aseton dan etanol. Disamping itu, ujian kemautan udang brin dan asai MTT masing-masing membuktikan bahawa semua ekstrak kasar tidak toksik dengan nilai LC50 > 1000 ppm dan nilai CC50 > 30 g/mL. Disamping itu juga, semua ekstrak menunjukan kesan aktiviti antimalaria yang sederhana terhadap 3D7. Manakala ekstrak aseton dan akueus menunjukan aktiviti yang tidak aktif terhadap W2 (IC50 > 50 ug/mL). Walaubagaimanapun, apabila Semambu digabungkan dengan nano-perak (Semambu-AgNO3), ia menunjukan peningkatan aktiviti antimalaria sehingga 4 kali ganda terhadap 3D7 (IC50 = 8.815 ± 0.230 dan W2 (IC50 = 23.110 ± 0.088). Secara konklusinya, kajian ini telah membuktikan bahawa pokok Semambu dari Malaysia memiliki aktiviti antimalaria yang mengalakkan apabila digabungkan bersama zarah nano dan perlu dikaji dengan lebih lanjut sifatnya bagi mengatasi kerintangan klorokuin dalam rawatan malaria.

INVESTIGATION OF ETHANOL, ACETONE AND AQUEOUS *Azadirachta indica* A. Juss (NEEM) EXTRACTS AGAINST *Plasmodium falciparum*

ABSTRACT

Malaria is a public health concern, causing significant morbidity and mortality annually due to the emergence of drug-resistance parasites. Therefore, the need for novel antimalarial agents with novel mechanisms of action and broad therapeutic potential is critical. In this study, A*zadirachta indica* A. Juss (Neem), a long-used herbal remedy has been chosen due to the scarcity of data on Malaysian Neem on its antimalarial property. Thus, this study aimed to determine *in vitro* antimalarial activity of Malaysian's Neem leaves crude extracts against laboratory-adapted, sensitive (3D7) and chloroquine-resistance (W2) *P. falciparum* strains. At first, all the collected leaves were taxonomized by The Forest Research Institute Malaysia (FRIM) and further authenticated molecularly before extraction with ethanol, acetone and aqueous followed by a phytochemical screening of the extracts. DPPH radical scavenging is used to determine antioxidant activity before GC-MS profiling. The brine shrimp lethality test (BSLT) and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against the primary human umbilical vein endothelial cells (HUVEC) were used for toxicity assay. The antimalarial activity was determined using the malarial SYBR Green I fluorescence-based assay against both strains. The study was extended with a green synthesis of Neem-silver nanoparticles (AgNPs) and further evaluated on their antimalarial property. Our data showed that crude extract produced by acetone (15.55 ± 0.04%) and ethanol (9.20 ± 0.05%) using the soxhlet method produces more yield compared to aqueous extract (5.86 ± 0.06%). However, aqueous

extract contained the highest total phenolic and flavonoid content. Meanwhile, the brine shrimp lethality test and MTT assay showed all extracts are not toxic with LC50 > 1000 ppm and CC50 values > 30 g/mL, respectively. Whereas, all extracts showed a moderate antimalarial effect against 3D7. However, acetone and aqueous extract showed inactive antimalarial activity against W2 (IC50 > 50 ug/mL). On the other hands, when Neem combined with nano silver (Neem-AgNO3), it showed a 4-folds increment of antimalaria activity against 3D7 (IC50 = 8.815 ± 0.230) and W2 (IC50 = 23.110 ± 0.088). Thus, this study showed promising antimalarial activity of Malaysian Neem when combining with nanoparticle and should be further evaluated of its property to overcome the chloroquine resistance in malaria treatment.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria is one of the most devastating blood- borne parasitic disease transmitted by female Anopheles mosquitoes that belong to a Plasmodium species. The World Health Organization (WHO) reported in 2019 that the number of people who remain at risk is around 3.2 billion and estimated at 219 million new cases with more than 435 000 deaths in 2018 was reported (WHO, 2019). Therefore, various malaria prevention programs have been carried out including integrated insecticide management and antimalaria therapy resulting 1.5 billion million cases and 6 million deaths have been avoided globally in the period 2000–2019 (WHO, 2020). However, due to the outbreak of Covid-19 pandemic, the number of malaria deaths in 2019 was doubling compared to the 2018 (WHO, 2020).

The spread of Plasmodium strains resistance to antimalarial drugs, particularly chloroquine, has hampered the malaria elimination effort (Gandhi *et al*., 2018). Thus, recognizing the challenge, WHO has intellectually designed a Global Technical Strategy for Malaria, expanding from the year 2016 until 2030 to diminish global malaria mortality and incidence by no less than 90% through an initiative to enhance the process of control, prevention and elimination. Currently, there are five (5) species of malaria-causing Plasmodium in human; 1) *Plasmodium falciparum* (*P. falciparum*), 2) *Plasmodium vivax* (*P. vivax*), 3) *Plasmodium malariae* (*P. malariae*), 4) *Plasmodium ovale* (*P. ovale*) and 5) *Plasmodium knowlesi* (*P. knowlesi*).

Among the five, *P. falciparum* has been reported to be the most virulent and causes an increase in the mortality and morbidity rate of malaria infection per year,

particularly in developing countries (Adedosu *et al*., 2015). This is due to high parasitaemia and the unique ability of *P. falciparum* mature infected red blood cells (iRBC) to sequester on human vascular endothelial cells which can lead to severe malaria (Craig *et al*., 2012; Gray *et al*., 2003; Kyes *et al*., 2001).

According to WHO (WHO, 2018), Algeria maintained its malaria-free status and El Salvador and China reported zero indigenous cases. In addition, Iran, Suriname, Malaysia, Saudi Arabia, the Republic of Korea and Timor-Leste showed a reduction of cases in 2017 (WHO, 2018). However, the disease has resumed increasing in countries with the highest disease burdens such as sub-Saharan Africa and India. Furthermore, the emergence of parasite resistance to antimalarial drug and insecticide is the challenge arises to eliminate malaria disease. Therefore, there is a need to discover a new antimalaria drug to curb more death.

Plant sources have been used in many generations as an alternative to combat infectious diseases, particularly by indigenous people due to their high availability. One of the available approaches is exploration of empirically characterised antimalarial plants approaches for developing a new drug for early malaria treatment. In addition, it represents a viable strategy for the discovery of new druggable active compounds against the multi-stage parasite cycle (Abay *et al*., 2015). The link between medicinal plants and successful antimalarial drug discovery dated back to 1820 with the isolation of quinine from Cinchona bark (Achan *et al*., 2011) and continues to the current drug used, artemisinin-based combination therapy (ACT), which is a semisynthetic derivative of artemisinin isolated as the active compound of Artemisia annua.

In this study, *Azadirachta indica* A. Juss one of the most used medicinal plants which originates in India, has been chosen as a source of a drug as it has been widely reported to possess an analgesic, anticancer, antiviral, antimalarial, antibacterial, and antifungal (Adedosu *et al*., 2015; Ahmed *et al*., 2016) antioxidant, immunomodulatory anti-inflammatory, antiulcer, antimutagenic and anticarcinogenic (Biswas *et al*., 2002). In addition, many studies of Neem leaves extract have been focused on cancer as reported by Schumacher (2011), Neem extract possessed pro-apoptotic and anti-proliferative effects on cancer cells (Schumacher *et al*., 2011). The activities of this plant are suggested due to the presence of phenolic compounds in the plant (Hismath *et al*., 2011).

It was reported that plant family Meliaceae commonly possess remarkable antioxidant activities through the polyphenolic compounds which have been effective in the prevention of disease (Nahak and Sahu, 2010) and the ability to exhibit various pharmacological properties (Hismath *et al*., 2011). Thus, Neem was selected due to its chemical constituents of biologically active compounds, including flavonoids, alkaloids, phenolic compounds, triterpenoids, steroids, carotenoids, and ketones (Deshpande *et al*., 2014).

Neem has been used by Indian people as a source of Ayurvedic medicine for more than 4000 years (Pandey *et al*., 2014) and the tree herb is known as “Margosa tree”, Indian Lilac and also called “the village pharmacy” due to its versatility for healing (Prashanth and Krishnaiah, 2014). Meanwhile, in Malaysia, the tree is called Neem as veepelai in Tamil, ying lian in Mandarin and Daun semambu in the Malay population (Nishan and Subramanian, 2014). Neem is an evergreen, rapid-growing tree that belongs to the family of Meliaceae, a subfamily of Meloideae and tribe of

Melieae which is the most diverse and versatile trees of the tropics with immerse medicinal potential (Girish and Shankara, 2008).

It was reported that ethanol and methanol is the best and effective solvent for extracting phenolic contents from Neem because of its lowers toxicity properties and has been proven as an effective solvent in phenolic compound extraction (Nahak and Sahu, 2010; Perumal and Klaus, 2003). Besides that, ethanolic Neem leaf extract displayed neuroprotective effects by mitigating oedema development and modulating apoptosis signalling pathways (Bedri *et al*., 2013). In other studies, methanolic Neem leaf extract shows a strong effect on proinflammatory cell signalling and apoptotic cell death mechanisms in human cancer cell lines of leukaemia (Schumacher *et al*., 2011).

Acetone-water extraction is proven more efficient solvent when compared with water alone, which supported by Udeinya and college in 2004, demonstrated that acetone-water mixture is more efficient solvent than water alone for Nigerian Neem leaves which showed antiretroviral activity and antimalarial activity (Girish and Shankara, 2008; Udeinya *et al*., 2004). A previous study reported IRAB, a fractionated acetone-water extract has been showing safer medicinal properties (Anyaehie, 2009). Maturation of malaria parasites beyond the ring stage was completely halted referring to the morphological development due to the heat-stable acetone-water extract of Neem leaf (Udeinya *et al*., 2004). In addition, extracts from Nigerian Neem leaves have been reported earlier to possess antimalarial activities (Deshpande *et al*., 2014).

Other than investigating the effect of Neem on the drug-sensitive and drug-resistant parasite, this study also trying to explore the use of nanotechnology. Nanotechnology is an area of applied science and technology that explores and exploits the structure of particles between 1 to 100 nm in size. Because of the high potential of

silver nanoparticles (AgNPs) mediated plants for antimicrobial properties, this study aims to identify and characterize the potential of Malaysian Neem-AgNPs green synthesis for anti-plasmodial properties toward inhibition of chloroquine-sensitive (3D7) and chloroquine -resistance (W2) of *Plasmodium falciparum* strains.

1.2 The rationale of the study

The increasing emergence of the drug-resistant parasite to most of the available antimalarial drugs is a major global health issue that requires innovative strategies and underlined the importance of discovering new antimalarial drugs to combat the disease. A major source of new antimalarial drugs were the medicinal plants since they are readily available, cost-effective and show comparatively fewer side effects compared to synthetic drugs.

Azadirachta indica A. Juss is a famous plant of medicinal values that have been utilized traditionally all over the world as herbal remedies in the treatment of various ailments and was reported to have a broad spectrum of antimicrobial activities including antiparasitic action. This interesting finding leads us to the screening of the extracts on malaria parasites, especially on *P. falciparum*. Bedri *et al* (2013) had shown that ethanolic extract of neem leaves able to decrease oedema in cerebral malaria mice model and Udeinya *et al.,* 2004 showed acetone-water Neem leaves extract possessed anti-cytoadhesion activity by inhibits adhesion of malaria parasite-infected erythrocytes on human endothelial cell (Bedri *et al*., 2013; Udeinya *et al*., 2004).

Since some country shows good potential activities of Neem towards antimalarial. Thus, in this study, we investigate Malaysia Neem because until now, there is no available published research using Malaysia Neem for antimalarial activity. Hence, this study may provide additional data on the use of Neem, especially

Malaysian Neem as an antimalarial drug to drug-resistant parasites and the use of nanotechnology to enhance drug development.

1.3 The objective of the study

1.3.1 The general objective of the study

The overall goal of this study is to determine the antimalarial properties of Malaysian Neem using ethanol, acetone, and aqueous crude extracts, as well as synthesized Neem-silver nanoparticles (Neem-AgNPs) on chloroquine-sensitive (3D7) and chloroquine-resistance (W2), strains of *P. falciparum*.

1.3.2 Specific objectives of the study

The specific objectives are as follows:

1. To investigate the phytochemical constituents of the Malaysian Neem leaves crude extracts.
2. To determine the toxicity effect of all the Neem leaves crude extracts using brine shrimp toxicity test and human umbilical vein endothelial cell (HUVEC).
3. To determine the effect of all the Neem leaves crude extracts by ethanol, acetone and aqueous on *Plasmodium falciparum* (3D7 and W2).
4. To examine the effect of the synthesized Neem-AgNPs on *Plasmodium falciparum* (3D7 and W2) and its haemolysis effect.

1.4 Experimental design

The overall design of the study is represented in Figure 1.1. The collected plant was authenticated at first by molecular fingerprinting and physical characterization. The leaves were then extracted using the Soxhlet method with three different solvents of varying polarity (acetone, ethanol, and aqueous) to yield three distinct crude extracts. The yield percentage of the crude extracts were recorded. The extraction method is also known as the separation of a crude extract of phytochemicals from raw materials.

Thus, phytochemicals analysis of total phenolics and total flavonoids content in the Neem leaves crude extracts was carried out and determined using colorimetric methods; Folin-Ciocalteu for phenolics and aluminium chloride for flavonoids. The antioxidant assay using the DPPH method was performed to determine the antioxidant properties of all Neem leaves crude extracts. The leaves crude extracts at different concentrations were exposed with a free radical 2,2-diphenyl-1-picryl- hydrazyl-hydrate (DPPH) solution and the absorbance was measured at 517 nm to determine the percentage of scavenging activity and the inhibition concentration that requires to reduce 50% of free radical DPPH (IC_{50}) All the crude extracts were screened for their active compounds using GC-MS.

To evaluate the efficacy and toxicity of these plant products, the toxicity of Neem leaves crude extract was tested. One of the toxicity studies of Neem leaves crude extracts includes the brine shrimp lethality test (BSLT). Different concentrations of leaves crude extracts were evaluated for their toxicity for 24 hours on mature brine shrimps. The lethality concentration was calculated via the percentage of mortal shrimps, which kills 50% of the shrimp population (LC_{50}).

Another cytotoxicity study of the Neem leaves crude extracts was evaluated using a 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Different concentrations of Neem leave crude extracts were used as treatment against normal human umbilical vein endothelial cell (HUVEC) for 72 hours before the addition of tetrazolium MTT salt solution (final concentration of 0.4 mg/mL). At 570 nm, the absorbance was measured and analysed to determine the cell viability reduction by 50% (CC_{50}) from the concentration of leaves crude extracts used. As for the measurement of antimalarial activity from the Neem leaves crude extract, the malarial SYBR Green I fluorescence-based assay was used.

Predominantly at the ring stage, *P. falciparum* cultures (3D7 and W2 strains) were synchronised with sorbitol prior to treatment with the Neem leaves crude extracts for 48 hours at different concentrations. After treatment of 48 hours, the SYBR Green I solution (2× final concentration from 10 000× stock concentration) was added to the parasite suspensions. Determination of the inhibitory concentration of extracts that kills half of the parasite population (IC50) was measured and analysed through the excitation wavelength of 490 nm and emission wavelength of 530 nm.

Aqueous Neem leaves crude extracts was then selected for the synthesis of silver nanoparticles (AgNPs). The synthesized Neem-AgNPs were then characterized by using Ultraviolet-visible spectroscopy, Dynamic light scattering and scanning electron microscope observation. The anti-plasmodial activity of Neem-AgNPs against *P. falciparum* (strain 3D7 and W2) was assessed at different concentration in 96-well plates for 48 hours via the malarial SYBR Green I fluorescence-based assay and using Chloroquine (CQ) as a standard drug. The inhibitory concentration of the

Neem-AgNPs that kills half of the parasite population (IC_{50}) was determined using GraphPad Prism V7 Software.

A haemolysis assay was done to further investigate the level of cytotoxicity for the synthesized Neem-AgNPs extracts against ordinary human red blood cells. Different concentrations of Neem-AgNPs were used to treat washed erythrocytes (2% haematocrit) from healthy human blood groups (O^+). The percentage of haemolysis (%) was measured via the absorbance of haemoglobin in the supernatants at 450 nm.

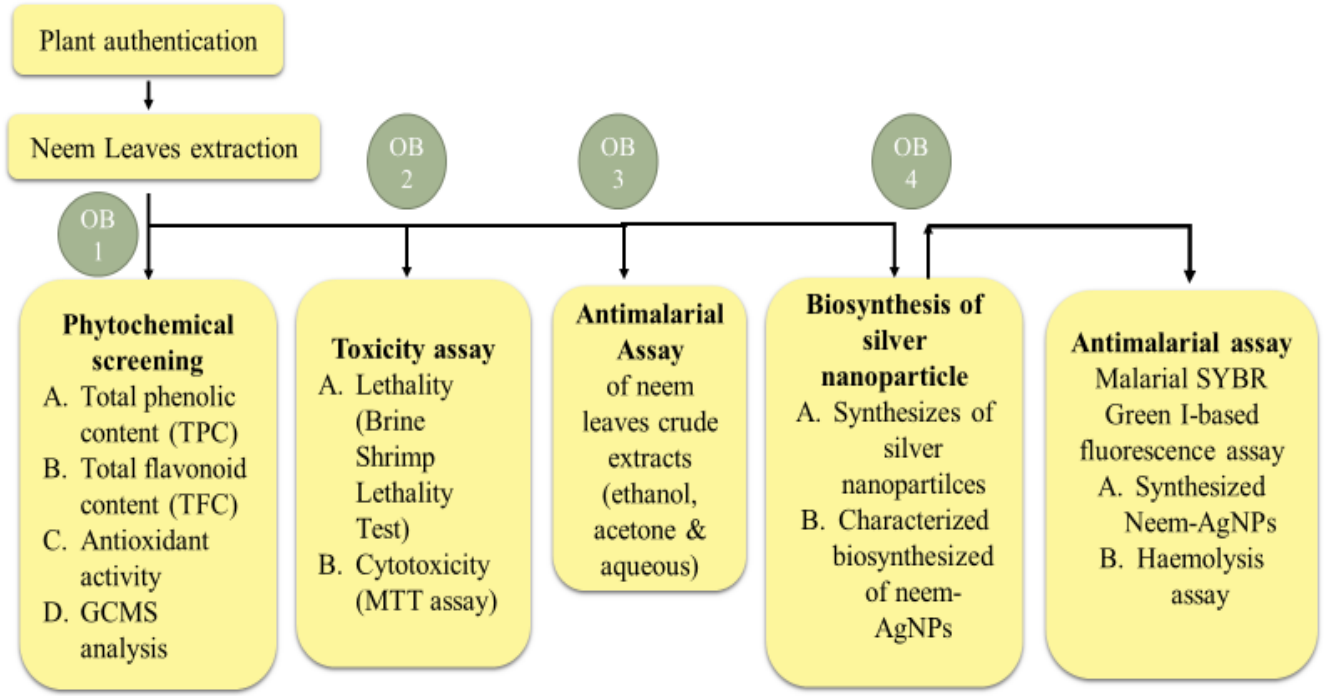

Figure 1.1 Flowchart of the experiments carried out through all the study

OB 1: Objective 1; OB2: Objective 2; OB3: Objective 3 and OB4: Objective 4

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of malaria

On Malaria is a blood-borne infectious disease transmitted from human to human via infected female Anopheles mosquitoes. This life-threatening disease caused by a Plasmodium genus currently remains an international hazard towards the process of human advancement especially in regions of tropical and subtropical countries (WHO, 2020). According to the world malaria report, estimated 229 million malaria cases in 2019 in 87 malaria-endemic countries with 409 000 death (WHO, 2020). The high vulnerability being among children under the age of five, pregnant women, a human with immunodeficiency and non-immune travellers (WHO, 2020).

Meanwhile, about 3% of malaria cases globally reported by WHO happen in the South-East Asia Region, which India contributed the most significant cases accounted for about 86% of all malaria deaths (WHO, 2020). Even though malaria incidence rates have declined in the last decade, they remain concerning due to the difficulty of interpreting changing disease transmission in a rapidly growing population, particularly in Africa (Venugopal *et al.*, 2020). Ninety-nine countries accounted for 95 per cent of all malaria cases worldwide which are Niger (3%), Mozambique (4%), Uganda (5%), the Democratic Republic of the Congo (12%), Nigeria (27%) and accounted for approximately 51% of overall cases worldwide (WHO, 2020). Figure 2.1 and Figure 2.2 show the latest world and Asia region maps of malaria death rates based on ages.

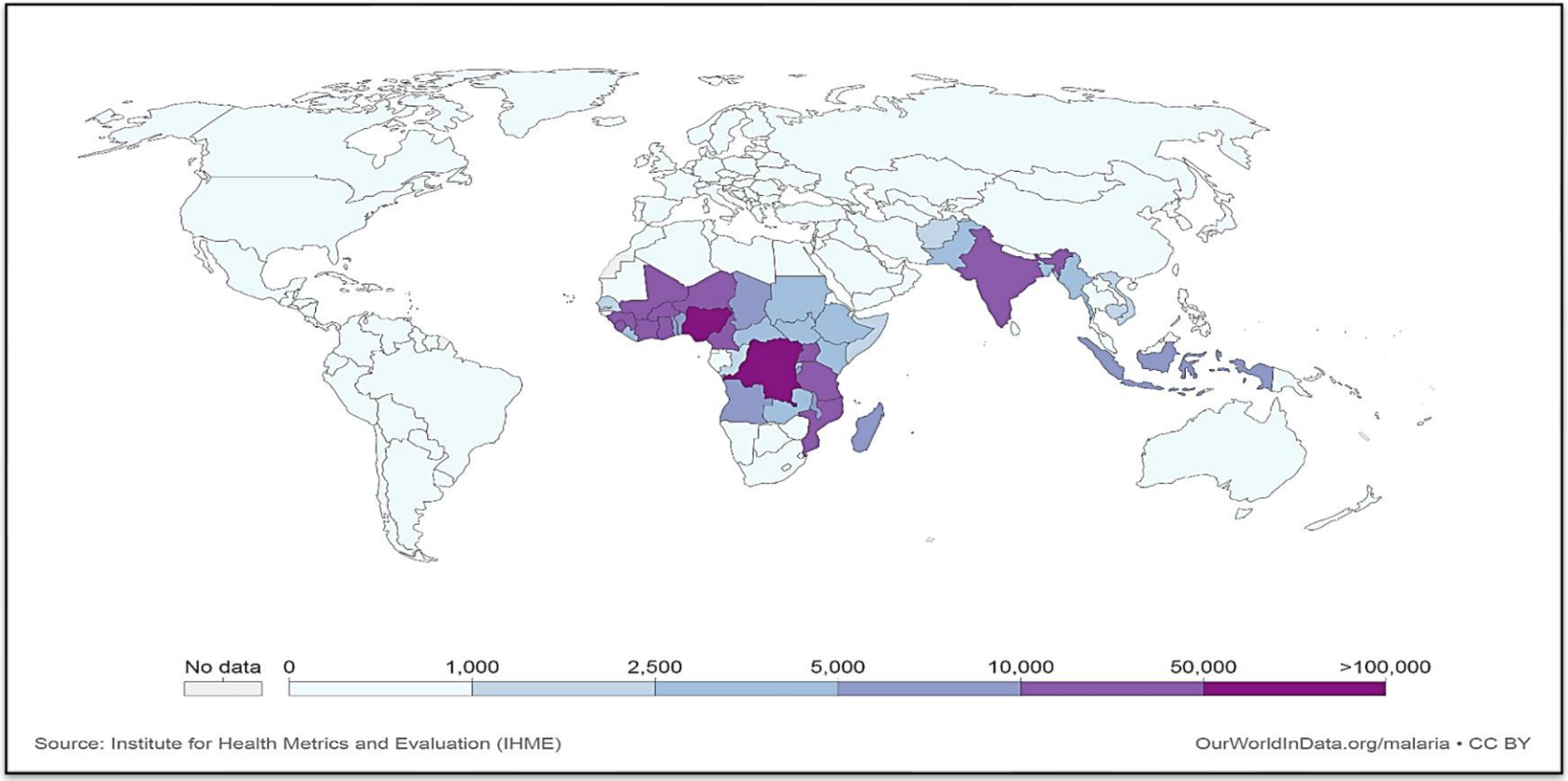

Figure 2.1 World malaria death rates, 2017.

Malaria death rates are age-standardized and expressed as the number of deaths per 10,000 people. The map was obtained from https://ourworldindata.org/malaria

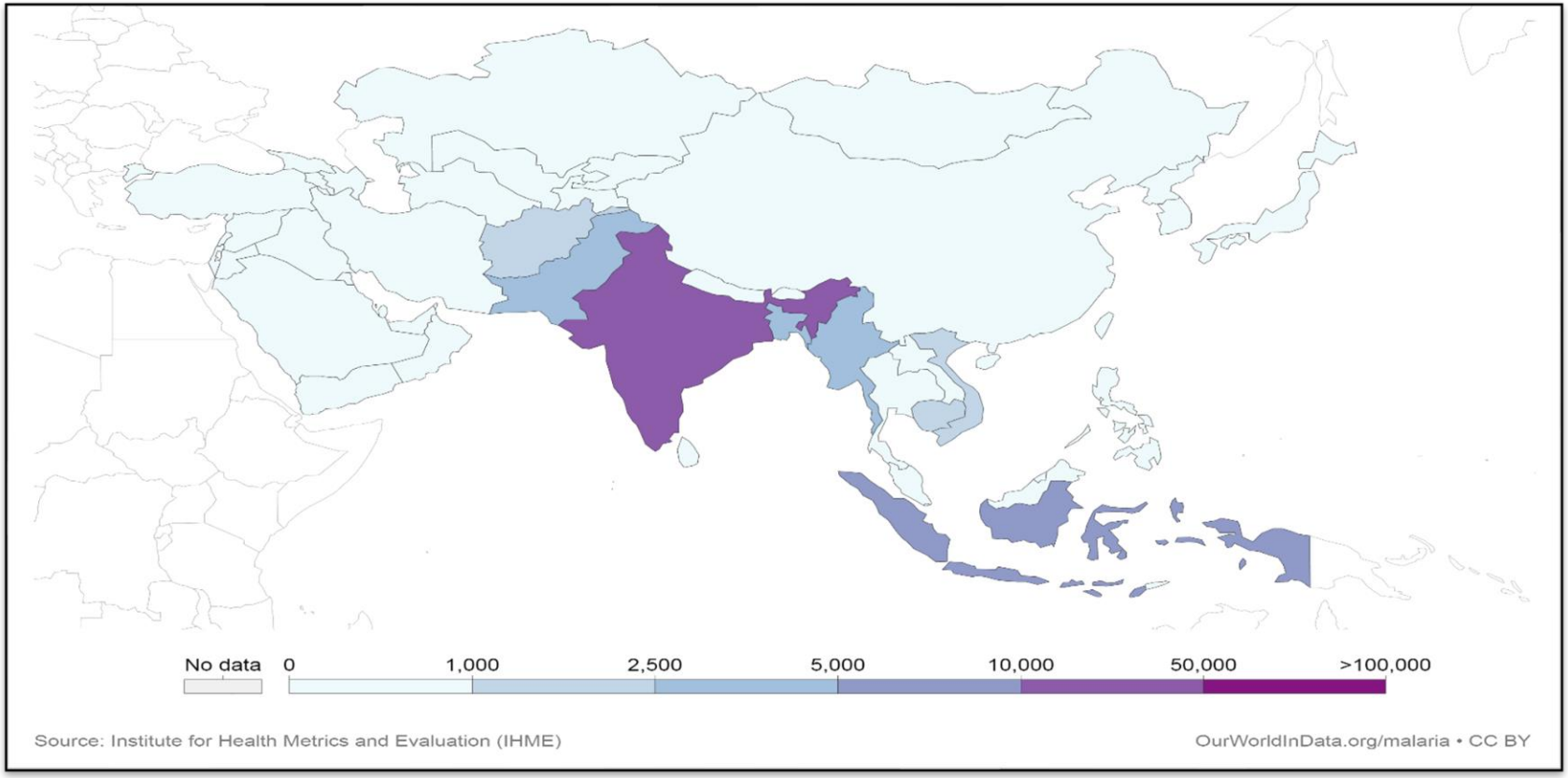

Figure 2.2 Asia malaria death rates, 2017.

Malaria death rates are age-standardized and expressed as the number of deaths per 100,000 people. The map was obtained from https://ourworldindata.org /malaria.

Five Plasmodium species that can cause human malaria, and there are *P. vivax, P. malariae, P. knowlesi, P. ovale* and *P. falciparum* (Tizifa *et al*., 2018). *P. vivax* is the most common species that cause malaria in non-African countries and recognized as one of the primary causes of human malaria in Malaysia (Tse *et al*., 2019). However, the emergence of zoonotic malaria caused by *P. knowlesi* that naturally occurs in macaques has elevated new malaria cases in Malaysia (Davidson *et al*., 2019; Lim *et al*., 2017; Singh *et al*., 2003). Meanwhile, *P. falciparum* is the most common malaria-causing parasite found in most African regions, accounting for the majority of malaria cases and deaths, and it is associated with a high risk of severe clinical manifestations (Talapko *et al*., 2019).

2.2 *Plasmodium falciparum*

P. falciparum is the most prevalent and lethal parasite infecting humans which can cause severe malaria and fatal (Avitabile *et al*., 2020). Most deaths caused by *P. falciparum* among African children are under the age of five (WHO, 2019). *P. falciparum* becomes the most extensively studied due to its unique ability to subvert the physiology of its host during the sexual stages of its development and its ability to adhere to the endothelial cell wall and sequester in small capillaries (Hanssen *et al*., 2010; Milner, 2018).

The infected red blood cell (iRBC) will have their surface modified by *P. falciparum* thus keeping the parasite out of circulation via the adhesive phenotype, for nearly half of its asexual life cycle, which is unusual among other malaria parasites (Emile *et al*., 2012). *P. falciparum* is not restricted to any region of the host and can infect a high proportion of red blood cells (RBCs), resulting in a high parasite burden, which linked to severe disease (Venugopal *et al*., 2020). Relapse cases in *P.*

falciparum infections have been observed, which can result in a rapid high parasitaemia with subsequent RBCs destruction (Chu and White, 2016; Saifi *et al*., 2010).

2.3 Life cycle of *Plasmodium falciparum*

To reproduce continuously, this human parasite life cycle requires two hosts: human red blood cells (asexual life cycle) and infected female Anopheles mosquitos (sexual life cycle). The process began during a blood meal of malaria-infected female *Anopheles* mosquito and continued in human RBCs (Figure 2.3).

2.3.1 The sexual life cycle of *Plasmodium falciparum*

During a blood meal, mosquito ingested male and female gametocytes from the infected host. The male gametocyte divides, within the mosquito midgut, into up to eight flagellated microgametes (ex-flagellation), whereas a single macrogamete was developed from the female gametocyte. In the mosquito midgut, the formation of a zygote happened after the fertilization of a macrogamete by a microgamete that undergoes meiosis. Then, it develops into an invasive and motile ookinete capable of penetrating the midgut wall.

The parasite asexually replicates when the ookinete forms an oocyst resulting in several thousand sporozoites (sporogony). Over time, the oocysts enlarge and release sporozoites after bursting which then renders the mosquito infectious to human beings due to their migration to the mosquito salivary gland where another blood meal will allow transmission back to the human host (Bousema and Drakeley, 2011). Mosquitoes take a blood meal to obtain nutrients for their egg production (Toenhake, 2020).

2.3.2 The asexual life cycle of *Plasmodium falciparum*

The *P. falciparum* life cycle began with asexual reproduction when an infected female Anopheles mosquito injected the human host with sporozoites during a blood meal (Talapko *et al*., 2019). When the sporozoites reach the liver, they develop into schizonts after they invade the hepatocytes and asexually replicate. After seven days of liver stage development, 40,000 merozoites were released by each infected hepatocyte into the peripheral bloodstream.

Once in the bloodstream, initiation of the replication cycle occurs after the merozoites quickly invade the circulating RBCs. At the schizont stage (schizogony), the parasite replicates into 8-32 daughter merozoites in which they previously progress through the ring and trophozoite stages within 48 hours. Mature asexual stages (trophozoites and schizonts) display the capability to increase RBCs stiffness, which enables them to be replenished by a spleen clearance. At this point, the merozoites were released into circulation when the parasitized RBC (pRBC) ruptures thus starting another cycle of asexual replication.

Instead of asexual replication, a small subset of parasites differentiates to produce sexual progeny in the following cycle which forms the gametocytes of male and female. A subset of parasites enters the extravascular space of the bone marrow after leaving the peripheral circulation, where gametocytogenesis occurs when the gametocytes mature and progress for eight to ten days thus becoming transmissible. Instead of the bone marrow which was suggested as the primary location of gametocyte maturation, the human body have also observed the presence of immature gametocytes elsewhere such as in the spleen.

During blood meals, male and female gametocytes were taken by the mosquitoes since they have re-entered the peripheral circulation. Early-stage gametocytes are sequestered, and only mature gametocytes circulate in the peripheral blood, where they can be taken up by mosquitoes and continue their sexual stages (Bousema and Drakeley, 2011).

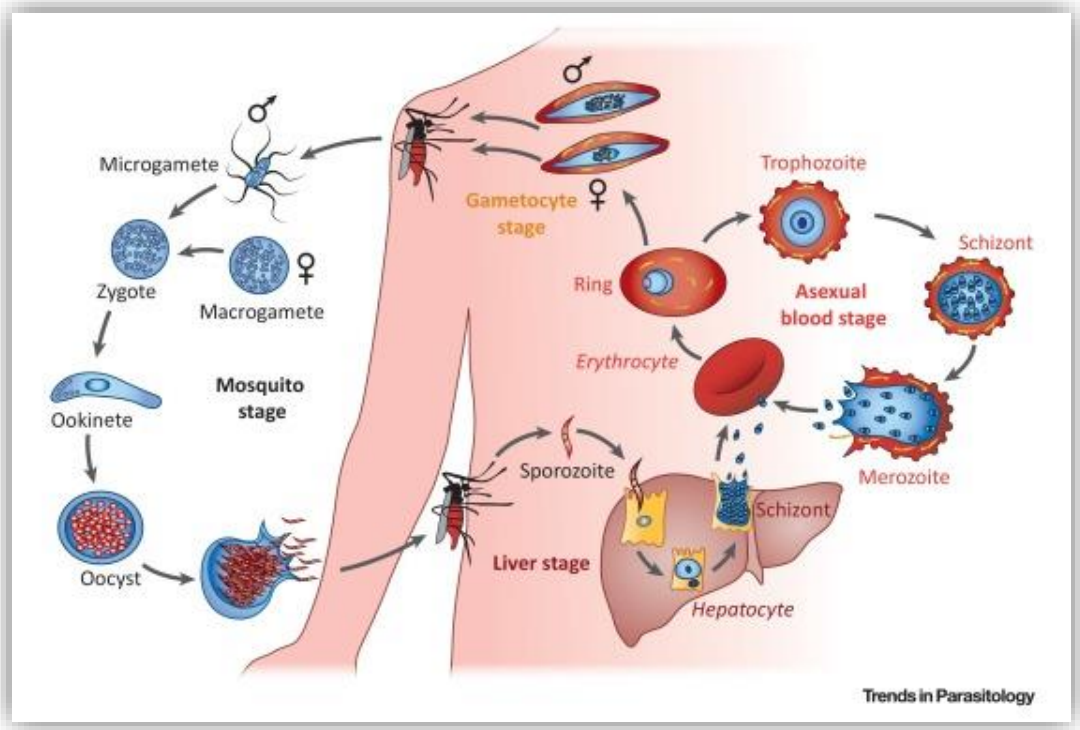

Figure 2.3 The asexual and sexual reproduction of *Plasmodium falciparum* within human and mosquito vector

Adapted from Maier *et al*., (2018)

2.4 Pathophysiology of *Plasmodium falciparum*

Plasmodium parasites got matured and reproduced in the liver after their journey through the bloodstream following Anopheles mosquitoes bite, which will later cause all the malaria typical symptoms such as fever, headache, pain, weakness, abdominal distress, nausea and excessive perspiration (Okello and Kang, 2019). Meanwhile, the signature fever-chill periodicity, headache, fatigue and nausea are among the early disease symptoms (Maier *et al*., 2018).

The "At-risk" population refers to the larger portion of individuals that were bitten by infected mosquitoes yet only shows positive malaria from the smear test diagnostic upon screening while they were asymptomatic (Stone *et al*., 2015). Malaria symptoms can only begin in any ill patient with the first liver schizont rupture and release of merozoites into the peripheral circulation, and for most patients, this event is silent (Milner, 2018).

Malaria can be categorized into two disease presentations which are uncomplicated and severe (Bartoloni and Zammarchi, 2012). Uncomplicated malaria usually come with symptoms of fever but an absence of any clinical or laboratory signs that can be related to the severity or vital organ dysfunction (WHO, 2015). It can be treated with antimalarial drugs during symptomatic occurrences, and most infected patients can clear the infection without worsening (Milner, 2018). However, in a non-immune person, mild symptoms such as fever, headache, and chills can be observed 10–15 days after infection. These symptoms are difficult to identify, but if not treated within 24 hours, they can lead to severe complications and death (WHO, 2020).

The adhesion of iRBCs to the cell lining of microvasculature is believed to be significant pathogenesis caused for severe malaria (Cojean *et al*., 2008). If a non-

immune or semi-immune host is infected with *P. falciparum* malaria, it can cause severe malaria (SM) with a higher risk of mortality if left untreated (Craig *et al*., 2012). Meanwhile, severe malaria frequently causes symptoms such as severe anaemia and respiratory distress due to metabolic acidosis, or cerebral malaria in children, but it is more likely to cause multi-organ failure in adults (WHO, 2019).

2.5 Malaria control, diagnostic and prevention challenges

Malaria control is the reduction of the disease burden to the point where it no longer poses a public health risk, whereas malaria elimination is the cessation of home-grown mosquito-borne malaria spread in a specific geographical area (WHO, 2008). In 2016, WHO has implemented the Global Technical Strategy (GTS) 2016-2030 intending to reduce malaria cases and death rates targeting at least 40% by 2020 followed by 75% and 90% by 2030 when compared to a 2015 baseline (WHO, 2020).

Significant progress has been made in reducing malaria cases where the transmission and control processes are complex to control and prevent malaria disease which is linked to a few factors such as environmental factors, political stability, public health infrastructure, and socioeconomic development (Toenhake, 2020). Current malaria control efforts mainly focus on eliminating the mosquitoes' breeding grounds and vector control measure, rapid diagnosis and treatment and chemoprophylaxis chemotherapy for infected individuals were taken to combat the disease (Ashley *et al*., 2018; Rahman *et al*., 2019).

Ngomane and Jager, use temephos, an organophosphate larvicide to control mosquito larvae on identified breeding sites (Ngomane and Jager, 2012). In an epidemic disease situation, the majority of malaria vector control programs concentrated on areas with the highest transmission and morbidity and mortality,

particularly in rural areas (Gueye *et al*., 2016; Walker, 2002). Combating the ongoing progression of insecticide resistance, discovering natural plant compounds, and developing longer-lasting insecticide formulations remain top priorities (The malERA Refresh Consultative Panel on Tools for Malaria Elimination, 2017).

The development of resistance against insecticide (e.g. dichlorodiphenyltrichloroethane, DDT) by the disease vectors was concerned (Bekono *et al*., 2020). Currently, pyrethroid-PBO nets and neonicotinoid insecticides for insecticide resistance are two vector control options that should be considered as part of an insecticide resistance management strategy (IRS) (Tizifa *et al*., 2018; WHO, 2020). Pyrethroid permethrin and the synergist piperonyl butoxide (PBO) were used to combat pyrethroid resistance in mosquitos (Martin *et al*., 2021).

Meanwhile, neonicotinoids are being developed for use in IRS as a component of insecticide resistance management to improve the performance of insecticidal control (Ngwej *et al*., 2019). Meanwhile, in the 1990s, malaria Rapid Diagnostic Tests (RDTs) targeting parasite antigens in patient blood were introduced as histidine-rich protein 2 (HRP2) expressed by *P. falciparum*, while Plasmodium lactate dehydrogenase (pLDH) expressed by all human malaria species (Cunningham *et al*., 2019; Moody, 2002; Mouatcho and Goldring, 2013). However, the ubiquity of *P. falciparum* histidine-rich protein 2 (pfHRP2) deletions in Africa has rendered the RDT ineffective in that region (WHO, 2020).

The current vaccine candidate RTS, S/AS01 has shown low to modest efficacy in preventing clinical malaria by *P. falciparum* (Navneet *et al*., 2021). National regulatory authorities (NRAs) in Ghana, Kenya, and Malawi have approved the RTS, S/AS01 malaria vaccine for use in pilot areas, and the European Medicines Agency

(EMA) has given it a positive scientific opinion (WHO, 2021). However, before implementation, a safety assessment must be performed to ensure that the vaccine is as safe, promising, well-tolerated, and immunogenic as possible (Cowman *et al*., 2016).

The World Health Organization was looking into the use of malaria medicines for chemoprevention in order to reduce severe disease and death among main target 15 groups including new-borns, children under the age of five, and pregnant women (WHO, 2020). The discovery of plant-based novel chemotherapeutic agents is well-known because they are rich in various active compounds that have the potential to treat a variety of diseases, including malaria (Erhirhie *et al*., 2021).

Proguanil is used in conjunction with chloroquine as chemoprophylaxis against *P. falciparum* malaria in areas with low resistance, such as tropical Africa (Abdi *et al*., 2003). In another study, a single dose of Atovaquone-proguanil revealed prophylactic efficacy in a human malaria challenge at time points relevant to weekly dosing schedules and post-exposure prophylaxis (Deye *et al*., 2012). Meanwhile, Mefloquine and atovaquone are recommended chemoprophylaxis drugs for non-immune travellers, and doxycycline is currently recommended chemoprophylaxis drugs for non-immune travellers to some disease-endemic countries around the world (Quashie *et al*., 2013). However, drug and insecticide resistance, social and economic, ethnicity, religious, and psychosocial beliefs and practices, and unreformed health care system have the potential to disturb the progress that has been made so far (Dhiman, 2019). To overcome the drawback of those, treatment measures have been reinforced using antimalarial drugs.

2.6 Antimalarial drugs and their limitations

Antimalarial medications are categorised based on their chemical structure and pharmacologic mechanism of action (Sevene *et al*., 2010). The major conventional drug used currently to treat malaria includes quinoline-related compounds, antifolates, artemisinin derivatives (peroxidase) and antibiotics (Tetracycline, doxycycline, clindamycin, azithromycin, fluoroquinolones) (Goodman *et al*., 2007).

Quinoline-based, such as chloroquine (CQ), hydroxychloroquine (HCQ), quinine (QN), mefloquine (MFQ), and quinacrine (QNX) are standard treatments that used to treat P. *falciparum* parasites (Pinheiro *et al*., 2019). Meanwhile, CQ, QN, and MFQ have been shown to inhibit acid proteases of *P. falciparum in vitro*, which involved in the digestion of ingested host cell cytosol inside the parasite's acidic food vacuole, but only at extremely high drug concentrations (Famin and Ginsburg, 2002; Gyang *et al*., 1982; Nqoro *et al*., 2017). Quinoline drugs work against malaria by interfering with heme detoxification (Foley and Tilley, 1998; Herraiz *et al*., 2019). However, their utmost mode of action is uncertain, and more research is necessary to understand how these medications may cause parasite toxicity to improve therapeutic efficacy and reduce resistance(Herraiz *et al*., 2019; Kumar *et al*., 2007).

Antifolate medicines like pyrimethamine (Pyr) and proguanil, the precursor of cycloguanil had already long been used in traditional malaria infections, particularly those caused by *P. falciparum* (Yuvaniyama *et al*., 2003). The drugs work by inhibiting the dihydrofolate reductase activity of the *P. falciparum* enzyme dihydrofolate reductase–thymidylate synthase (PfDHFR-TS), and resistance develops as the enzyme's binding affinities with the inhibitors deteriorate (Cowman *et al*., 1988). In 1945s, Proguanil has been reported as one of the early antifolate antimalarial drugs

(Curd *et al*., 1945; Tse *et al*., 2019) and in 1991s the drugs have been reported used for the treatment of protozoan infections (Hudson *et al*., 1991; Tse *et al*., 2019).

The emergence of universal CQ resistance resulted in its replacement as first-line therapy in many parts of the world by anti-folate drugs, most notably sulphadoxine-pyrimethamine (SP) (Sridaran *et al*., 2010). However, in many settings, artemisinin combination therapy (ACT) has been introduced to either work in conjunction with anti-folates or to replace them as first-line therapies(Nduati *et al*., 2008; Wongsrichanalai *et al*., 2002).

However, no single drug capable of eradicating all stages of the parasite's life cycle has been discovered or manufactured yet. As a result, single or combination classes of drugs are frequently administered concurrently to combat malarial infection synergistically. Treatment is based on several factors, including the geographic location of the infection, the type of Plasmodium species infected, and the severity of disease presentation in the patients. Figure 2.4 shows distribution of chloroquine and artemisinin resistance of *Plasmodium falciparum* over the world.

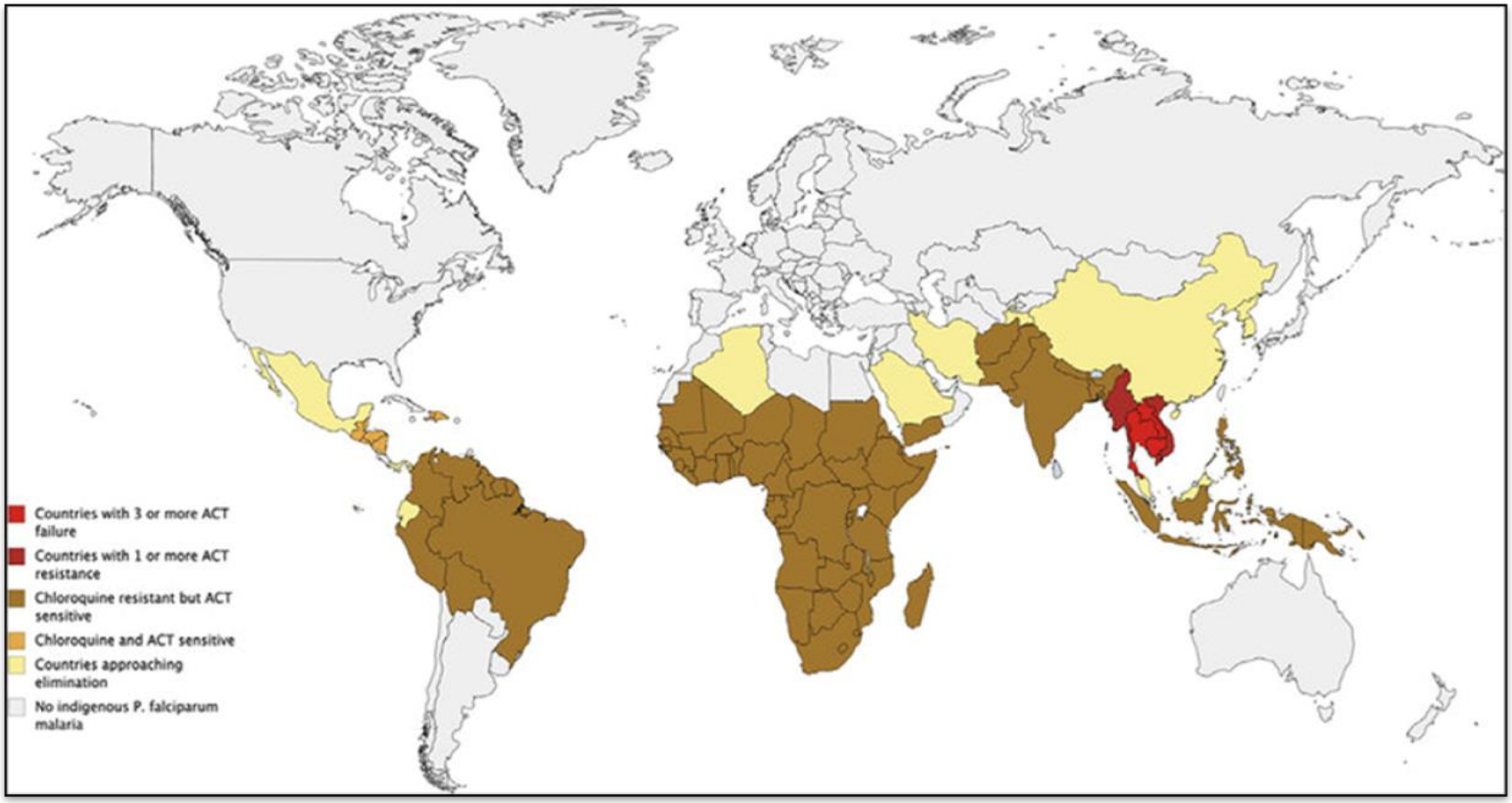

Figure 2.4 Global distribution of chloroquine and artemisinin resistance of *Plasmodium falciparum*.

Adapted from (Plewes and Leopold, 2019).